PATENT COOPERATION TREATY

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PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant s or agent s file reference 300400RU	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/RU 2003/000474	International filing date (day/month/year) 05 November 2003 (05.11.2003)	Priority date (day/month/year) 12 November 2002 (12.11.2002)

International Patent Classification (IPC) or national classification and IPC

C12N 15/11,15/10,15/63,15/82,A01K 67/00,A01H 1/00,C07K 14/435,16/18,C12Q 1/68, G01N 33/533

Applicant

ZAKRYTOE AKTSIONERNOE OBSCHESTVO "EVROGEN" et al

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This Report consists of a total of 4 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claimes and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under PCT).

These annexes consist of a total of 7 sheet

3. This report contains indications relating to the following items:

I ☒ Basis of the report

II ☐ Priority

III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

IV ☐ Lack of unity of invention

V ☒ Reasoned statement under Articl 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

VI ☐ Certain documents cited

VII ☐ Certain defects in the international application

VIII ☐ Certain observations on the international application

Date of submission of the demand: 19 May 2004 (19.05.2004)	Date of completion of this report: 03 March 2005 (03.03.2005)
Name and mailing address of the IPEA/ RU FIPS Russia, 121858, Moskva, Berezhkovskaya nab., 30-1 Facsimile No.	Authorized officer M.Kuptsova Telephone No 240-25-91

Form PCT/IPEA/409 (cover sheet)(July 1998)

EXPRESS MAIL LABEL NO.: EV 480459479 US

INTERNATIONAL PR[redacted]INARY EXAMINATION REPORT

International application No.
[redacted]/RU 2003/000474

I. Basis of the report

1. With regard to the elements of the international application:*

☐ the international application as originally filed

☒ the description:
pages 1-26, 28-31, as originally filed
pages 27, filed with the demand
pages _____, filed with the letter of

☒ the claims:
pages _____, as originally filed
pages _____, as amended (together with statement) under Article 19
pages _____, filed with the demand
pages 32-34, filed with the lette 21.12.2004

☒ the drawings:
pages 1/20-20/20, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of

☒ the sequence listing part of the description:
pages 1, 5-17, as originally filed
pages 2, 3, 4, filed with the demand
pages _____, filed with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1.(b)).
☐ the language of publication of the international application (under Rule 48.3(b)).
☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☒ contained in the international application in written form.
☒ filed together with international application in computer readable form.
☐ furnished subsequently to this Authority in written form.
☐ furnished subsequently to this Authority in computer readable form.
☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:
☐ the description, pages _____
☐ the claims, Nos. _____
☐ the drawings, sheets/fig. _____

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No
PCT/RU 2003/000474

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-24	YES
	Claims		NO
Inventive Step (IS)	Claims	1-24	YES
	Claims		NO
Industrial Applicability (IA)	Claims	1-24	YES
	Claims		NO

2. Citations and explanation:

The examination report is established for amended claims in view of the following documents:

D1 – 1GFL A. Chain A, Structure of Green Fluorescent Protein
D2 – JP 10-234382;
D3 – US 6232107;
D4 – US 5976796;
D5 – WO 1997/041228;
D6 – CA 2331882.

D1 discloses a primary structure of green fluorescent protein isolated from an organism belonging to the class Hydrozoa having an amino acid sequence, which is about 50% identical to SEQ ID NO:2 characterizing the nucleic acid according to claim 1.

D2 discloses information about a nucleic acid sequence encoding green fluorescent protein, a vector comprising thereof, host cells capable of synthesizing the indicated fluorescent protein. D2 also provides possibility for the use of the obtained protein as a labeling agent for detecting the protein localization in live cells, as a reporter for the analyses of promoters, etc.

D3 discloses primers and probes capable of hybridizing with nucleic acid sequence encoding green fluorescent protein having the length of 14 n.

D4 discloses a green fluorescent and luciferase fusion protein. D4 also discloses a method of making monoclonal antibodies to said protein, a method of making the protein and the possibility of its use as a double marker for monitoring gene expression in living cells and quantitatively by enzymatic activity.

D5 discloses a plant comprising a nucleic acid molecule encoding green fluorescent protein included in expression vector.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

D6 discloses a mouse comprising an expression system including a nucleic acid encoding green fluorescent protein.

The Applicant has restricted claims 1 and 14 by indicating the minimum possible length of the protein fragment comprising 15 amino acid residues.

Although an amino acid sequence of the fluorescent protein disclosed in D1 is about 50% identical to the sequence shown in SEQ ID NO:2, it does not comprise fragments having 15 amino acid residues and more, which completely coincide in structure with the claimed protein fragments. Hence, features of claims 1 and 14 are not known from D1 and D2-D6.

Consequently, claims 1-4 and 14 meet the criterion of novelty.

The presence of fluorescent proteins in medusas of the genus Aequorea gives grounds for search of similar proteins in organisms belonging to other genera but related to the same class Hydrozoa. However, none of the retrieverior art documents teaches that fluorescent proteins of medusas of different genera can have the homology attaining 50%, which makes it possible to use the nucleic acid encoding green fluorescent protein as a tool for the isolation of DNA encoding proteins with similar properties from organisms belonging to Anthomedusae. Hence, an isolated nucleic acid according to claims 1-4 and a protein according to claim 14 and the use thereof for labeling molecules, cells, etc. is not obvious and requires an inventive activity. Based on the foregoing claims 1-4 and 14 meet the criterion of inventive step.

Claims 5-13, 15-24 also meet the criteria of novelty and inventive step, since they contain features of claims 1 or 14.

Claims 1-24 meet the criterion of industrial applicable.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule, which encodes a fluorescent or chromo- protein, selected from the group consisting of:

(a) a nucleic acid which encodes a protein comprising the amino acid sequence as shown in SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22;

(b) a nucleic acid comprising a nucleotide sequence as shown in SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21;

(c) a nucleic acid that hybridizes under stringent conditions to the nucleic acid of (a) or (b) above;

(d) a nucleic acid that encodes a protein that has at least about 75% sequence identity to the amino acid sequence of (a) above;

(e) a nucleic acid that has at least about 70% sequence identity to the nucleotide sequence of (b) above;

(f) a nucleic acid which encodes a protein having at least one amino acid substitution, deletion or insertion in the amino acid sequence as shown in SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22.

(g) a derivative or mimetic of the nucleic acid of (a), (b), (c), (d), (e) or (f) above;

(h) a mutant of the nucleic acid of (a), (b), (c), (d), or (e) above;

(i) a nucleic acid which differs from the nucleic acid of (b), (c), (d), (e), (f), (g) or (h) above due to the degeneracy of genetic code; and

(j) a fragment of the nucleic acid of (a) or (b) above encoding a peptide of at least 15 amino acid residues in length.

2. The nucleic acid molecule of claim 1, wherein said nucleic acid is isolated from an organism from a Class Hydrozoa.

3. The nucleic acid molecule of claim 1, wherein said nucleic acid is isolated from an organism from a Sub-order Anthomedusae

4. The nucleic acid molecule of claim 1, wherein said nucleic acid is isolated from a Genus *Phialidium.*

5. A vector comprising the nucleic acid molecule according to claim 1.

6. An expression cassette comprising (a) the nucleic acid molecule according to Claim 1; and (b) regulatory elements for the expression of said nucleic acid molecule in the desired host-cell.

7. A cell comprising the nucleic acid molecule according to claim 1, the vector according to claim 5, or the expression cassette according to claim 6.

8. A stable cell line comprising the nucleic acid molecule according to claim 1, the

vector according to claim 5, or the expression cassette according to claim 6.

9. A transgenic plant comprising the nucleic acid molecule according to claim 1, the vector according to claim 5, or the expression cassette according to claim 6.

10. A transgenic animal comprising the nucleic acid molecule according to claim 1, the vector according to claim 5, or the expression cassette according to claim 6.

11. A method for producing a fluorescent or chromo- protein, said method comprising (a) providing a nucleic acid molecule according to claim 1 operably linked to suitable expression regulatory elements (b) expressing the fluorescent or chromo- protein from said nucleic acid molecule, and (c) isolating the protein substantially free of other proteins.

12. A nucleic acid molecule comprising a fragment of the nucleic acid molecule according to claim 1, said fragment encoding a peptide of at least 100 amino acids in length

13. A nucleic acid molecule having a sequence that is substantially the same as, or identical to a nucleotide sequence of at least 300 residues in length of the nucleic acid molecule according to claim 1.

14. An isolated fluorescent or chromo- protein selected from the group consisting of:

(a) a protein comprising the amino acid sequence as shown in SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22;

(b) a protein encoded by the nucleic acid molecule comprising a nucleotide sequence as shown in SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21;

(c) a protein that has at least about 75% sequence identity to the amino acid sequence of (a) or (b) above;

(d) a mutant of the protein of (a), (b) or (c) above;

(e) a protein having at least one amino acid substitution, deletion or insertion in the amino acid sequence as shown in SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22.

(f) a derivative of the protein of (a), (b), (c), (d) or (e) above;

(g) a fragment of the protein of (a), (b), (c), (d), (e) or (f) above comprising of at least 15 amino acid residues in length; and

(h) a protein having a sequence that is substantially the same as, or identical to the amino acid sequence of at least 100 residues in length of (a) or (b) above.

15. A fusion protein comprising the protein according to claim 14.

16. An antibody specifically binding to the protein according to claim 14.

17. A kit comprising the nucleic acid according to claim 1, the vector according to claim 5, the expression cassette according to claim 6, the protein according to claim 14, the fusion protein according to claim 15, or a means for producing the same.

18. An oligonucleotide probe or primer comprising the nucleotide sequence capable of

hybridizing to the nucleotide sequence selected from the group consisting of SEQ ID NOs. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21.

19. A method for labeling a biological molecule, comprising coupling said biological molecule to the protein according to claim 14.

20. A method for labeling a cell comprising production of the protein according to claim 14 in the cell.

21. A method for labeling a cell organelle comprising production of the protein according to claim 14 fused to the suitable subcellular localization signal in the cell.

22. A method for analyzing a biological molecule, cell or cell organelle comprising detection of fluorescence signal from the protein according to claim 14 or 15.

23. A method for analyzing a biological molecule, cell or cell organelle comprising expression of the nucleic acid molecule according to claim 1 in a cell.

24. A method of detecting a biological molecule comprising detection of fluorescence signal from the protein according to claim 14 or 15.

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Val His Val Pro Glu Tyr His His Ile Thr Tyr His Val Thr Leu Ser
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        35                  40                  45
Thr Thr Gly Asp Val Pro Val Pro Trp Ser Thr Leu Val Thr Thr Leu
    50                  55                  60
Thr Tyr Gly Ala Gln Cys Phe Ala Lys Tyr Gly Pro Glu Leu Lys Asp
65                  70                  75                  80
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115 120 125
Lys Lys Asp Gly His Val Leu Gly Lys Asn Leu Glu Phe Asn Phe Thr
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Pro His Cys Leu Tyr Ile Trp Gly Asp Gln Ala Asn His Gly Leu Lys
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180 185 190
Val His Val Pro Glu Tyr His His Met Thr Tyr His Val Thr Leu Ser
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65                  70                  75                  80
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Glu Asn Gly Ser Val Tyr Asn Arg Val Lys Leu Asn Gly Gln Gly Phe
        115                 120                 125
Lys Lys Asp Gly His Val Leu Gly Lys Asn Leu Glu Phe Asn Phe Thr
    130                 135                 140
Pro His Cys Leu Tyr Ile Trp Gly Asp Gln Ala Asn His Gly Leu Lys
145                 150                 155                 160
Ser Ala Phe Lys Ile Arg His Glu Ile Thr Gly Ser Lys Gly Asp Phe
                165                 170                 175
Ile Val Ala Asp His Thr Gln Met Asn Thr Pro Ile Gly Gly Gly Pro
            180                 185                 190
Val His Val Pro Glu Asn His His Met Ser Tyr His Val Lys Leu Ser
        195                 200                 205
Lys Asp Val Thr Asp His Arg Asp Asn Met Ser Leu Lys Glu Thr Val
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Arg Ala Val Asp Cys Arg Lys Thr Tyr Leu
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atgagctacc atgtcaagct cagcaaagat gttactgatc acagggataa catgagcttg     660
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phiYFP-M1 using mammalian-optimised codons (SEQ ID NOs: 09, 10, and 27). "Humanized" version of phiYFP-M1 was subjected for site directed and random mutagenesis to obtain green and cyan light emitting versions of the protein. Mutant fluorescent proteins with green and cyan fluorescence were obtained. The green mutant of the humanized phiYFP-M1, named phiYFP-M1G1, contained the following amino acid substitutions (as compared with phiYFP-M1): T65S, L148Q, Y203T, K231T, T232A (SEQ ID NOs: 17, 18, and 31). The cyan mutant of the humanized phiYFP-M1, named phiYFP-M1C1, contained the following amino acid substitutions (as compared with phiYFP-M1): L6Q, T65S, Y66W, N124K, C147Y, L148Q, Y203T, V224L (SEQ ID NOs: 19, 20, and 32). Excitation-emission spectra for this protein are shown at Figure 3A,B.

Example 3

hydr1GFP cloning, sequencing and recombinant protein production

Bright green fluorescence was detected using a fluorescent microscope in a hydromedusa 1 (about 1 mm in length, Figure 4) of sub-order *Anthomedusae* (*Cnidaria, Hydrozoa, Anthomedusae*). To search for the gene responsible for the fluorescence in this jellyfish, a strategy based on screening of an expression cDNA library in *E. coli* was implemented. Amplified cDNA samples were prepared using a SMART cDNA amplification kit (Clontech) and cloned into the PCR-Script vector (Stratagene). About 10^5 recombinant clones were screened visually using a fluorescent stereomicroscope. Three fluorescent clones were identified, each encoding the same green fluorescent protein, which was named hydr1GFP. The nucleotide and amino acid sequences for this protein are shown in SEQ ID NOS: 11, 12, and 28. A comparison of hydr1GFP with *A. victoria* GFP is shown in Figure 1. hydr1GFP appears to be more similar to GFP (37% identity) than to fluorescent proteins from corals.

To facilitate protein purification, the coding region of hydr1GFP was cloned into pQE30 expressing vector (Qiagen), so that recombinant protein contained six-histidine tag at its N-terminus. After expression in *E. coli*, hydr1GFP was purified by the metal-affinity resin, TALON (Clontech). The excitation-emission spectra for hydr1GFP showed peaks at 474 nm and 494 nm (Figure 5). In contrast to wild type *A. victoria* GFP, the novel hydr1GFP protein possessed only one absorption-excitation peak, which may correspond to a deprotonated chromophore state.

Example 4

hm2CP cloning, sequencing and recombinant protein production

Bright green fluorescence was detected in small hydromedusa 2 of sub-order *Anthomedusae* (*Cnidaria, Hydrozoa, Anthomedusae*) using fluorescent microscope. To search for FP from this jellyfish we chose a strategy based on screening of expression cDNA